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#25

Patent
Attorney's Docket No. P-015-RP1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	
	)	
Ju-Hua JI et al.	)	Group Art Unit: 1639
	)	
Application No.: 09/456,429	)	Examiner: M. Garcia Baker
	)	
Filed: December 8, 1999	)	
	)	
For: NOVEL CALCIUM CHANNEL DRUGS AND USES	)	

DECLARATION OF JACQUELINE ANNE McINNES SMITH PURSUANT TO 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Jacqueline Anne McInnes Smith, Ph.D., hereby declare the following:

1. I hold the position of Associate Director in the Biochemistry Dept. at Theravance, Inc. I am presently the Group Leader for the GPCR/Ion Channel Assay Development Team, a role that I have held since starting at Theravance in November, 1999 as a Senior Scientist.

2. I have a B.Sc. in Biochemistry, with First Class Honors from the University of Bath, in Bath, U.K.; a Ph.D. in Biochemistry from St. John's College, University of Cambridge, in Cambridge, U.K.

3. I am a co-author of 16 peer-reviewed published journal articles, and 14 abstracts.

4. The twenty-four compounds listed in the attached EXHIBIT A were synthesized in Theravance's laboratories and were assayed under my direction by the procedures described in EXHIBIT B.

5. The Fluorometric Imaging Plate Reader system (FLIPR) assay is well known as a validated assay for measuring calcium channel activity. See, for example, Denyer et al., *Drug Discovery Today* 3(7):327-328 (1998) (enclosed as Attachment A). Data results from the in-house T-type calcium channel FLIPR assays were further validated by close correlation of patch clamp assay data and FLIPR data for eight known standard pharmaceutical compounds used as calcium channel blockers.

6. The ability of these representative compounds to block the L-, N- and T-type calcium channels were determined under my direction using the FLIPR assays described in EXHIBIT B. The pIC_{50} values determined for these compounds are shown in EXHIBIT A.

7. In the FLIPR assays described in EXHIBIT B, compounds having higher pIC_{50} values have stronger inhibitory activity against the calcium channels.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 13th May 2003

J A M Smith

Jacqueline Anne McInnes Smith, Ph.D.

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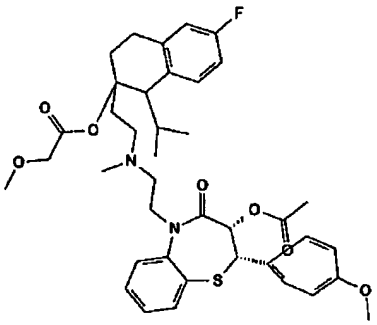
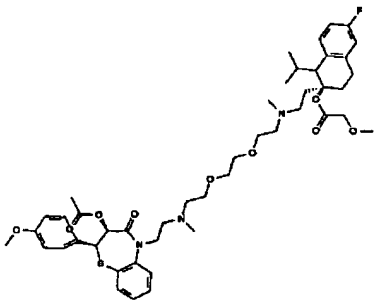
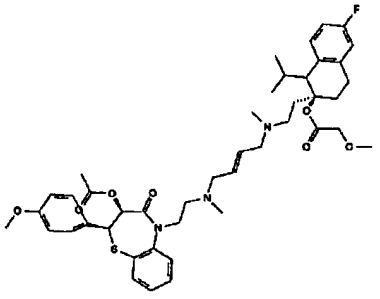
Calcium Channel FLIPR Assays

Tracking number	Compound Number in Table*	structure	L-Type FLIPR pIC50	N-Type FLIPR pIC50	T-Type FLIPR pIC50
1	#186		4.9		5.6
2	#200		5.5	5.2	5.5
3	#196		5.6		5.6

*"Compound Number in Table" refers to compound number in table appended to the specification as filed.

Calcium Channel FLIPR Assays

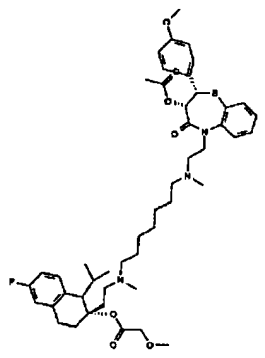
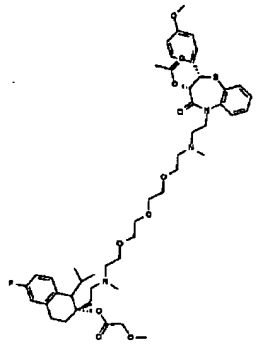
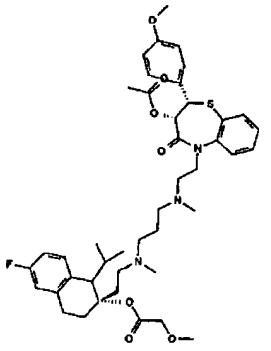
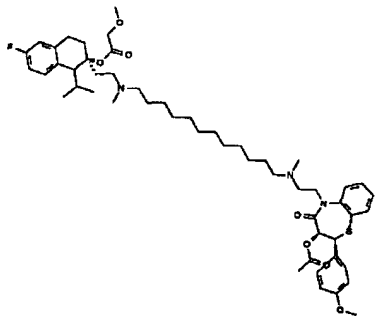
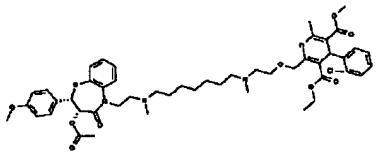
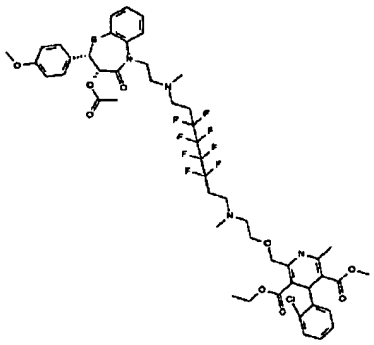
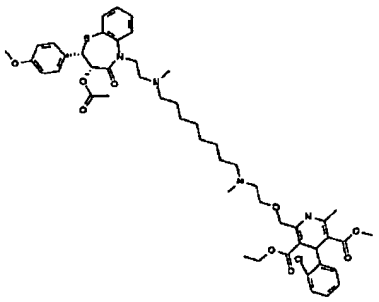
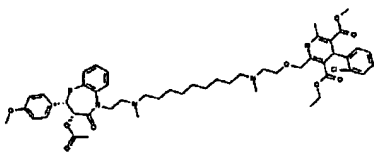
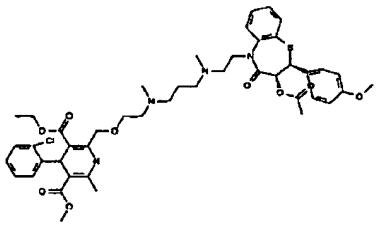
Tracking number	Compound Number in Table*	structure	L-Type Ft IPR pIC50	N-Type FLIPR pIC50	T-Type FLIPR pIC50
4	#192		6	5.3	5.7
5	–		5.3	5.1	5.7
6	#188		5.6		5.7

Exhibit A
Application S rial No. 09/456,429

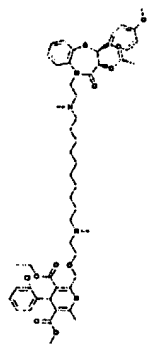
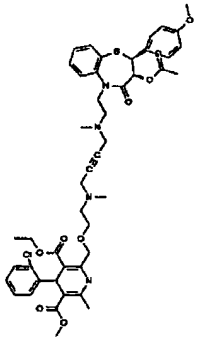
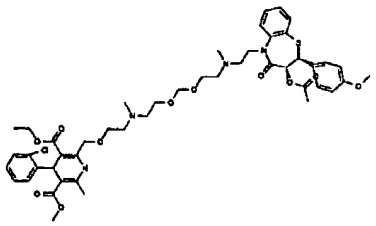
Calcium Channel FLIPR Assays

Tracking Number	Compound Number in Table*	Structure	L-Type FLIPR pIC50	N-Type FLIPR pIC50	T-Type FLIPR pIC50
7	--				5.9
8	#386		5.6		
9	#412		5.4		

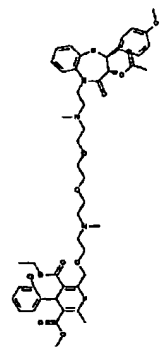
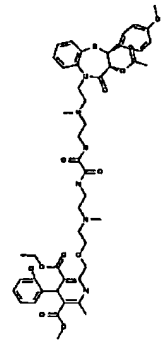
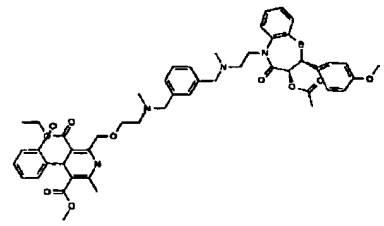
Calcium Channel FLIPR Assays

Tracking number	Compound Number in Table*	structure	L-Type FLIPR pIC50	N-Type FLIPR pIC50	T-Type FLIPR pIC50
10	#387		5.3		
11	#388		5.6		
12	#382		5.5		5.4

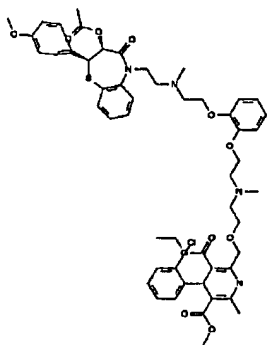
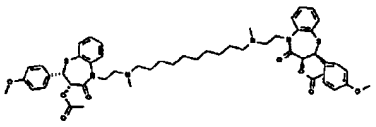
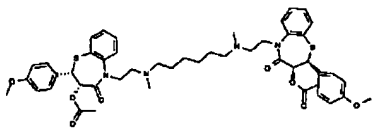
Calcium Channel FLIPR Assays

Tracking number	Compound Number in Table*	Structure	L-Type FLIPR pIC50	N-Type FLIPR pIC50	T-Type FLIPR pIC50
13	#389		5.7		5.9
14	#392		5.7		5.6
15	#411		5.9		5.5

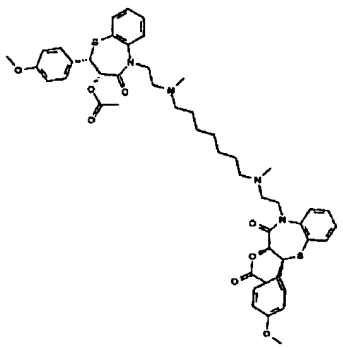
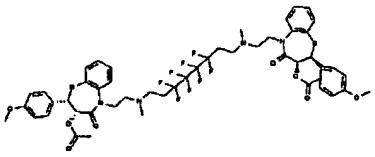
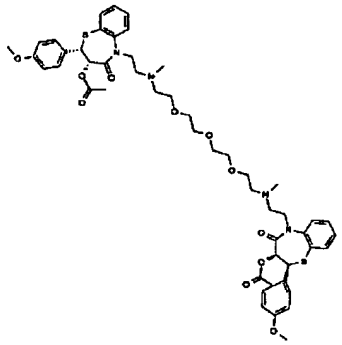
Calcium Channel FLIPR Assays

Tracking number	Compound Number in Table	structure	L-Type FLIPR pIC50	N-Type FLIPR pIC50	T-Type FLIPR pIC50
16	#394		5.7		5.7
17	#416		5.4		5.9
18	#407		5.4		6

Calcium Channel FLIPR Assays

Tracking number	Compound Number in Table*	structure	L-Type FLIPR pIC50	N-Type FLIPR pIC50	T-Type FLIPR pIC50
19	--		5.3		6.1
20	--		5.1		5.5
21	--		5.4		5.4

Calcium Channel FLIPR Assays

Tracking number	Compound Number in Table1	structure	L-Type FLIPR pIC50	N-Type FLIPR pIC50	T-Type FLIPR pIC50
22	--		4.9		
23	--		5.5		
24	--		5.6		5.5
		average value	5.5	5.2	5.7
		# of compounds assayed	23	3	18
		total	120.3	15.6	102.2

Inhibiti n of L-, N- and T-type Calcium Channel Mediated Increases in Intracellular Calcium Using FLIPR Assays

Abbreviations not defined below have their generally accepted meaning. Also, unless noted otherwise, starting materials, mediums, reagents and solvents were purchased from commercial suppliers (such as Gibco, Sigma and the like) and were used without further purification.

DMEM	Dulbecco's Modified Eagle's Medium
MEM	Minimum Essential Medium
FBS	Fetal Bovine Serum
FLIPR	Fluorometric Imaging Plate Reader
HBSS	Hank's Buffered Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEK	Human Embryonic kidney cells
SHSY5Y cell line	Human Epithelial cell line, ATCC accession no. CRL-2266

The purpose of these assays was to measure the effects of proprietary compounds on calcium channels via fluorometric measurement of changes in intracellular calcium concentration using a FLIPR instrument.

The FLIPR (Molecular Devices, Sunnyvale, CA) assay uses a calcium sensitive dye that fluoresces when free calcium binds. This fluorescence event is measured in real time by the FLIPR, which detects the change in fluorescence from a monolayer of cells.

Since each of the three types of calcium channels assayed differ not only by their function, but also by their sensitivity to pharmacological agents, separate FLIPR assays were developed to evaluate representative compounds of the invention by comparison with known industry standards for their ability to modulate calcium entry through each of the L-, T- and N-type calcium channels respectively. The L- and N-type assays have been described previously in the literature. See, for example, Morton et al., *Molecular Brain Research*, 13:53-61 (1992), and Hu et al., *Bioorg. Med. Chem. Lett.*, 9(15):2151-2156 (1999). The T-type assay was developed in house and was validated by close

correlation of in-house patch clamp assay data and T-type FLIPR data collected for eight known standard compounds using the T-type FLIPR assay protocol described herein.

Different cell lines were used for each of the different types of calcium channels. HEK 293 cells transfected with the human alpha1H ($Ca_v 3.2$) calcium channel were used for the T-type assay; SH-SY5Y neuroblastoma cells were used for the L-type calcium channel assay; and IMR-32 human neuroblastoma cells were used for the N-type calcium channel, respectively.

Potency for N- and L-type channels was determined by the ability of test compounds to inhibit increases in intracellular calcium evoked upon depolarization with high extracellular K^+ concentration. Potency for the T-type calcium channel was determined by the ability of test compounds to inhibit increases in intracellular calcium evoked upon elevation of extracellular Ca^{2+} concentration.

The protocol for the L-type calcium channel FLIPR assay is described in detail first, followed by a brief description of how the protocols for the N-type and T-type assays differed from the L-type protocol described herein.

L-type Calcium Channel FLIPR Assay Protocol

Cell Culture and Preparation

Cells were incubated in a 95% O_2/5% carbon dioxide, humidified incubator at 37 °C.

For the L-type calcium channel assay, SHSY5Y cells were grown in a media consisting of 50% MEM containing Earle's salts and 0.1 mM non-essential amino acids (Gibco#10370) supplemented with 2 mM L-glutamine (Gibco#25030), 1 mM sodium pyruvate (Gibco#11360) and 50% F12 Ham containing 2 mM L-glutamine (Gibco#11765). The 50/50 mixture was supplemented with 10% FBS (Gibco#10437) and (100 units)penicillin -(100 µg)streptomycin/mL (Gibco#15140).

Prior to each assay run, the cells were harvested by the following steps: the media was aspirated; cells were rinsed with 10 mL Ca^{2+} and Mg^{2+} free PBS, then the PBS was aspirated and the cells were briefly rinsed with 10 mL Versene (Gibco #15040). The cells were dislodged from the flask with light tapping and 10 mL of

fresh growth media were added. Cells were either frozen for later use or seeded for assays.

To prepare frozen stocks, cells were harvested from the 50-75% confluent flasks and pelleted at 1000 rpm for 5 minutes. The pellets were resuspended in 1 mL growth media/5% DMSO per 75 cm^2 of surface area. Cell suspensions were aliquoted 1 mL/tube into cryo-vials and frozen at 1 degree C/min in an insulated box in a -80 °C freezer. After 24 hours, cryo-vials were transferred to liquid nitrogen storage. To thaw a frozen stock, the cells were rapidly thawed in a 37 °C water bath for no more than 2 minutes. The thawed suspension from a cryo-vial was seeded into a T-75 flask containing 25 mL growth media. After the cells started to adhere to the side of the flask, the media in the flask was replaced to remove any remaining cryo-preservative.

FLIPR Assay

For FLIPR assays of calcium channel activity, the cells were aliquoted into black-walled, clear bottom 96 well microtiter plates coated with poly-D-lysine (Becton/Dickinson #354640) at a density of 30,000 cells/well. 100 µl of media were added to each well, followed by a 100 µl solution containing the appropriate number of cells mixed with media. Cells were incubated at room temperature for 30 minutes after seeding and then were incubated for at least 1 hour in a 95% O_2/5% CO_2 incubator at 37 °C before dye loading. Seeded cells were washed twice with FLIPR buffer[1] to remove growth media leaving 50 µl/well of FLIPR buffer, using a Cellwash (MTX Labsystems, Inc.). The cells were then incubated with an additional 50 µl/well of 4 µM FLUO-3AM (Molecular Probes, Eugene, OR) (a 2X solution was made) for 60 minutes at 37 °C, 5% carbon dioxide. The final dye concentration was 2 µM. Additionally, 1.5 mM probenecid (final concentration) was included in the buffer (only for the L-type assay). Following the dye incubation period, cells were washed 2-3 times with FLIPR buffer, leaving a final volume of 50 µl/well.

[1] The FLIPR buffer for the L-type assay was 1X Hank's buffered saline solution (Gibco #14185), 2 mM calcium chloride, and 10 mM HEPES. For the N-type assays, FLIPR buffer was 140 mM sodium chloride, 5 mM potassium chloride, 10 mM glucose, 2 mM calcium chloride, and 10 mM HEPES. For the T-type assay, the FLIPR buffer was: 140 mM sodium chloride, 5 mM potassium chloride, 10 mM glucose, 0.5 mM calcium chloride, and 10 mM HEPES.

Test compounds were diluted to 5 mM in dimethyl sulfoxide followed by a 1:100 dilution into FLIPR buffer. Dilutions were done using a Biomek 2000 robot with a pre-set software program. The Biomek robot prepared six 1:10 dilutions of the test compound.

After washing out the dye, 50 µl of diluted test compounds were added to the plate and the plate was incubated for about 5 minutes at room temperature on the FLIPR. A 10 second baseline fluorescence reading was followed by the addition of 100 µl stimulus buffer[2] to each well, then fluorescence was measured for 3-4 minutes at 2 second intervals to capture the maximum fluorescence change.

The parameters used for the FLIPR were: exposure time of 0.4 seconds, laser strength of 0.5 watts, excitation wavelength of 488 nm, and emission wavelength of 550 nm.

The change in fluorescence was expressed as maximum fluorescence minus baseline fluorescence for each well. The raw fluorescence data were plotted against the logarithm of drug concentration and fitted using nonlinear regression, with GraphPad Prism (GraphPad Software, Inc., San Diego, CA), to the following algorithm formula:

$$Y = \text{bottom} + (\text{top-bottom})/(1+10\Lambda((pIC_{50}+X)*\text{Slope})).$$

For each batch of cells used to assay test compounds, a standard compound with a known FLIPR pIC_{50} value (i.e., Mibefradil for the T-type, nicardipine for L-type assays; w-conotoxin MVIIC (Sigma #C4188) or MVIIA (Sigma #C1182) for the N-type assay) was tested to ensure the validity of the assay.

Difference in Protocol for N-Type Calcium Channel Assay

For the N-type calcium channel assay, IMR-32 human neuroblastoma cells were grown in medium consisting of MEM containing Earle's salts and non-essential amino acids (Gibco#10370), supplemented with 10% FBS (Gibco#10437), 1mM sodium

[2] Stimulus buffer used for L-type assay was: 100 mM potassium chloride, 45 mM sodium chloride; 10 mM glucose, 10 mM HEPES, and 2 mM calcium chloride, pH 7.4. For N-type assay, stimulus buffer was 5 mM sodium chloride, 140 mM potassium chloride, 10 mM glucose, 2 mM calcium chloride, and 10 mM HEPES, pH 7.4. For T-type assay, stimulus buffer was 140 mM sodium chloride, 5 mM potassium chloride, 10 mM glucose, 20 mM calcium chloride, and 10 mM HEPES, pH 7.4.

pyruvate (Gibco#11360), 2 mM L-glutamine (Gibco#25030), and (100 units)penicillin-(100 µg)streptomycin/ml (Gibco#15140). Cells were grown to 50% confluency and then differentiated for seven days in medium consisting of MEM containing Earle's salts and non-essential amino acids, supplemented with 1% FBS, 1mM sodium pyruvate, 2 mM L-glutamine, penicillin-streptomycin, 1mM dibutyryl cAMP (Sigma#D-0627), and 10uM 5-bromo-2'-deoxyuridine (Sigma#B-9285). Differentiation medium was changed every 48 hours.

Cells were aliquoted into 96 well microtiter plates at a density of 50,000 cells per well. Compounds were diluted in compound buffer[3] containing 10 µM nitrendipine or nifedipine to block residual L-type channel signals in IMR-32 cells. The Biomek robot prepared six 1:3 dilutions of the test compound.

Difference in Protocol for T-Type Calcium Channel Assay

For the T-type calcium channel assay, Human Embryonic kidney cells (HEK) stably expressing alpha 1H (Ca_v 3.2) T-type calcium channel (from L. Cribbs, Loyola Univ. IL) were grown to near confluency in medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) containing 4,500 mg/L D-glucose and pyridoxine hydrochloride (Gibco #21068) supplemented with 10% Fetal Bovine Serum (FBS) (Hyclone #SH30070.03), 1 mg/mL Geneticin (Gibco #10131), 4 mM L-glutamine (Gibco#25030), (100 units)penicillin -(100 µg) streptomycin/mL (Gibco#15140) and 0.5 mM calcium chloride. Two days before the T-type assay was run, cells were split to approximately 25% confluency. Cells were given fresh media one day prior to the assay. When the cells were harvested, instead of 10 mL Versene, cells were rinsed with 5 mL 0.25% trypsin-EDTA (Gibco#25200) then the trypsin solution was aspirated.

Cells were aliquoted into 96 well microtiter plates at a density of 35,000 cells per well and incubated at least 3 hours in a 95% O_2/5% carbon dioxide, humidified incubator at 37 °C before dye-loading. FLUO-4AM fluorescent dye was used instead of FLUO-3AM dye. The Biomek robot prepared six 1:3 dilutions of the test compound.

---

[3] Compound buffer was mixed as follows: 140 mM sodium chloride, 5 mM potassium chloride, 10 mM glucose, 2 mM calcium chloride, 10 mM HEPES, and 10 µM nitrendipine or nifedipine.

The data analysis was slightly different for this assay. Instead of measuring the maximum fluorescence minus the baseline fluorescence for each well as was done in the L- and N-type assays, in this assay, the difference between the baseline signal and the final fluorescence value (endpoint) was calculated, which was then further manipulated using the GraphPad algorithm formula in order to calculate the pIC_{50} value.

Results

A complete list of data results for representative proprietary compounds tested in the herein described assays is attached in Exhibit A. Representative compounds of the invention demonstrated pIC_{50} values summarized in the table below.

Compounds	L-type Assay	N-type Assay	T-type Assay
Compounds 1-24			
Range of results	4.9-6.0	5.1-5.3	5.4-6.1
Average	5.5	5.2	5.7
Control standards			
Mibefradil	5.5	5.1	6.0
Flunarizine	5.2	4.7	5.7
Nitrendipine	7.5	--	5.1
MVIIC (conotoxin)	--	7.8	--
MVIIA (conotoxin)	--	7.8	--

Drug Discovery Today
research focus

ATTACHMENT A

HTS approaches to voltage-gated ion channel drug discovery

Jane Denyer, Jennings Worley, Brian Cox, Gary Allenby and Martyn Banks

Voltage-gated ion channels are emerging as a target class of increasing importance to the pharmaceutical industry because of their relevance to a wide variety of diseases in the cardiovascular, CNS and metabolic areas. In the quest to identify novel lead molecules against these targets, drug discovery programmes are increasingly making use of HTS approaches. The authors describe the current technologies available for voltage-gated ion-channel screening, their application to HTS campaigns and the current limitations and emerging technologies within this area.

Voltage-gated ion-channel modulators currently represent a multi-billion pound worldwide market for the treatment of cardiovascular and CNS disorders. Given this success, together with the emerging links between disease and channel dysfunction, and the progress in molecular and functional characterization of the ion-channel families, voltage-gated ion channels are being actively pursued as targets for a wide variety of diseases in the CNS, cardiovascular and metabolic areas. Recent listings of voltage-gated ion-channel compounds nearing or at clinical development reflect an ever growing level of investment in voltage-gated ion-channel R&D[1].

Voltage-gated ion channels play a critical role in shaping the electrical activity of neuronal and muscle cells, and in controlling the secretion of neurotransmitters and hormones through the gating of calcium ion entry. Large families of voltage-gated sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) ion channels have been defined using electrophysiological, pharmacological and molecular techniques[2,3]; they are named according to their selective permeability for a particular cation with reference to their voltage dependence, kinetic behaviour or molecular identity (Table 1).

Although the structures of Na^+, Ca^{2+} and K^+ channels are quite different, there are common functional elements represented in each (Figure 1). The channels are all transmembrane proteins with an ion-selective aqueous pore that, when open, extends across the membrane[2]. Channel opening and closing ('gating') is controlled by a voltage-sensitive region of the protein containing charged amino acids that move within the electric field. The movement of these charged groups leads to conformational changes in the structure of the channel resulting in conducting (open/activated) or nonconducting (closed/inactivated) states. These ion-channel states provide unique opportunities for drug discovery, enabling state-dependent molecules to be developed that, for example, only bind to nonconducting (inactivated) channels. The overall effect is to target drugs to tissues exhibiting abnormal electrical activity, while leaving normal channels in active tissues unaffected.

Designing high-throughput screens for voltage-gated ion channels requires a different approach than for drug targets such as 7-transmembrane receptors or ligand-gated ion

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Table 1. Voltage-gated ion-channel families

Subunit structure	Nomenclature and tissue distribution	Molecular identity	Disease targets
Ca^{2+} channel		**Of α1 subunit**	**For Ca^{2+}-channel blockers**
Subunits: α1, β, γ, α2–δ	P/Q-type (neuronal)	α1A	Angina, arrhythmia, hypertension, atherosclerosis, pain, neuroprotection, migraine, stroke, Alzheimer's, cognitive enhancement and dementia
	N-type (neuronal)	α1B	
	L-type (cardiac)	α1C	
	L-type (neuronal)	α1D	
	R-type (neuronal)	α1E?	
	T-type (neuronal)	α1G	
	L-type (skeletal)	α1S	
	T-type (cardiac)	?	
Na^+ channel		**Of α subunit**	**For Na^+-channel blockers/ modulators**
Subunits: α, β1, β2	Brain I	BI	Epilepsy, pain, anaesthesia, neuroprotection, arrhythmia and migraine
	Brain II	BII, BIIA	
	Brain III	BIII	
	Brain VI	BVI	
	Peripheral neuronal 1	PN1	
	Sensory neurone-specific	SNS (PN3)	
	Glial	NaG	
	Skeletal muscle 1	SKM1	
	Heart/skeletal muscle 2	SKM2	
K^+ channel*	**Delayed rectifier (Kv family)**	**Of α subunit**	**For K^+-channel blockers**
Subunits: α (tetramer forms pore), β1, β2, β3 (not shown)	Brain/heart/skeletal muscle	Kv1.1	Multiple sclerosis, Alzheimer's, arrhythmias, immunosuppression and depression
	Brain/heart	Kv1.2	
	Brain/lung	Kv1.3	
	Brain/heart/skeletal muscle	Kv1.4	
	Brain/heart/kidney/skeletal muscle	Kv1.5	
	Brain	Kv1.6	**For K^+-channel openers**
	Brain	Kv1.7	Angina, hypertension, asthma and urinary incontinence
	Brain/heart/kidney/skeletal muscle	Kv2.1	
	Brain/heart	Kv2.2	
	Brain/lymphocytes/skeletal muscle	Kv3.1	
	Brain	Kv3.2	
	Brain/liver	Kv3.3	
	Brain/skeletal muscle	Kv3.4	
	Brain	Kv4.1	
	Brain/heart/aorta	Kv4.2	
	Brain/lung	Kv5.1	
	Brain	Kv6.1	
	Ca^{2+}-activated K^+ (BK)		
	Smooth muscle/brain	hslo	
	Other		
	Heart	herg	

*Only K^+ channels with S4 voltage-sensitive domain are presented in this table.

channels, because of the nature of the activation process. As mentioned above, voltage-gated ion channels do not require agonist binding for activation. Hence, there is no physiologically relevant agonist binding site, but rather sites that have been identified experimentally to be important in enabling conformational transitions of channels from conducting to nonconducting states. Such sites may be exploited in drug discovery programmes.

Techniques used in building high-throughput ion-channel assays include functional and radioligand-binding approaches applied to cells (vesicles or membranes) expressing native or cloned channels, or to whole-cell assays. Functional whole-cell assays may use electrophysiological techniques, such as patch clamping (which provides a controlled voltage stimulus and direct electrical readout of ionic current), or may make use of toxins, venoms or compounds that bind to and open channels (see Table 2). In the latter cases the kinetics of ion flux through open channels can be measured using fluorescence, end-point radiotracer or cell viability techniques.

The challenge posed in designing and running HTS campaigns lies in translating these assays into low cost, miniaturized, high-density formats. Typically, screening programmes are designed to assay 50,000–200,000 samples depending on the degree of automation and format (96- or 384-well) of the assay. Smaller campaigns tend to employ directed compound sets, selected around known ion-channel chemistries, whereas larger campaigns often use more diverse or random compound selection. Hence, the ability to perform highly automated, high-density assays increases the chances of discovering and exploiting completely novel chemical leads.

The following sections describe examples of ion-channel assays that have been reported in the literature or explored in-house at Glaxo Wellcome, and highlight technological developments in each area.

Techniques

Radioligand binding

Radioligand binding studies have revealed that voltage-gated ion channels possess distinct binding sites for different classes of channel activators and modulators[4]. For Na^+ channels, five neurotoxin binding sites have been well characterized[5] (Table 3) by either direct measurement of specific binding of labelled neurotoxin to the site or competitive displacement of a labelled neurotoxin by other unlabelled neurotoxins. Additional sites are also implicated

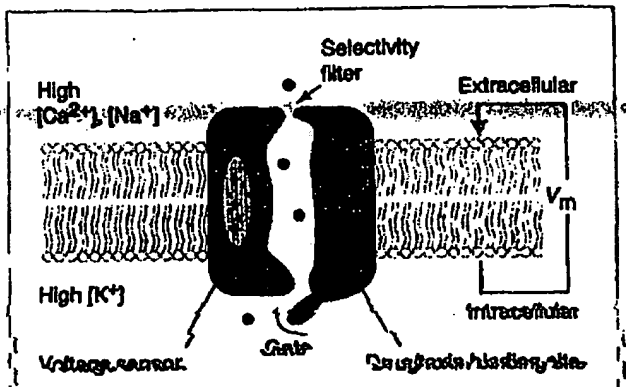

Figure 1. Schematic representation of a voltage-gated ion channel. All voltage-gated ion channels possess a positively charged region, which acts as a dipole detecting the electric field across the membrane. The pore contains a region that selectively favours passage of a particular ionic species (Na^+, Ca^{2+} or K^+). A gating mechanism controls the ionic conductance of the channel. Multiple binding sites for toxins and pharmacological agents are found on these channels – only in the case of BK Ca^{2+}-activated K^+ channels is a binding site known to be physiologically relevant in modulating the channel state. V_m, membrane potential.

for other classes of neurotoxins and compounds including insecticides, local anaesthetics and anticonvulsants[6].

For some ligands, the affinity for a particular site has been found to vary according to the channel state (open, closed or inactivated), and hence they exhibit voltage or state-dependent binding. For example, with Na^+ channels, lamotrigine, lidocaine and phenytoin bind preferentially to the ion channel in the inactivated state[6,7], whereas batrachotoxin and veratridine bind with high affinity to open channels[6]. Interestingly, lidocaine appears to competitively displace batrachotoxin, but the interaction does not take place through competition at site 2; it occurs through allosteric antagonism – by stabilizing the channel in the inactivated state when site 2 exhibits low affinity for batrachotoxin[6]. Such interactions between binding sites, the voltage-dependency of binding and the multiplicity of potential binding sites available on ion channels affecting function, inevitably means that great care is required in designing a meaningful high-throughput assay.

To design a binding assay for a voltage-gated ion channel, an ideal situation would be to identify a high-affinity

Tabl 2. Some toxicolocical/pharmacological agents for voltage-gated Ion-channel assays

Ion channel	Blockers	Activators
Ca^{2+} channel		
N-type	ω-Conotoxin MVIIA peptide toxin from *Conus magnus* and ω-conotoxin GVIA peptide toxin from *Conus geographus*	
P/Q-type	ω-Agatoxin-IVA (peptide from *Agelenopsis aperta*)	
Q-type	ω-Conotoxin MVIIC (peptide toxin from *C. magnus*)	
T-type	Mibefradil (anti-arrhythmic/antihypertensive)	
L-type	1,4-Dihydropyridines e.g. nimodipine (anti-arrhythmic/antihypertensive)	BAYK8644 (pharmacological tool)
Na^+ channel		
	Tetrodotoxin (heterocyclic guanidine toxin from *Tetradon stellatus* and other fish of the Tetraodontiformes order); local anaesthetics e.g. lidocaine; μ-conotoxin GIIIB peptide toxin from *Conus geographus*; anticonvulsants (e.g. lamotrigine, phenytoin); antiarrhythmics (e.g. quinidine, mexiletine)	Batrachotoxin (steroidal alkaloid from *Phyllobates aurotaenia*); aconitine (alkaloid toxin from *Aconitum* sp.); veratridine (alkaloid toxin from *Veratum* sp.); scorpion venom (from *Leiurus quinquestriatus*); ATXII (toxin from *Anthopleura xanthogrammica*); pyrethroids (insecticides) derived from *Chrysanthemum* sp.; brevetoxins (toxin from *Ptychodiscus brevis*)
K^+ channel		
Kv1.1, 1.2, 1.6	α-Dendrotoxin peptide from *Dendroaspis angusticeps*	
Kv1.3	Charybdotoxin peptide from *Leiurus quinquestriatus hebreaus*	
Kv1.3	Margatoxin from *Centruoides margaritatus*	
Kv1, Kv3, Kv4.2	4-Aminopyridine (pharmacological tool)	
Kv1.1, 1.6, 2.1, Kv3	Tetraethylammonium (pharmacological tool)	
(Ca^{2+}-activated K^+ (BK/hslo)	Paxilline alkaloid toxin from *Penicillium paxilli*	NS1608 (pharmacological tool)
	Iberiotoxin peptide from *Buthus tamulus*	

Table 3. Neurotoxin binding sites for Na^+ channels

Site	Toxin	Effect on Na^+ channel
1	Tetrodotoxin Saxitoxin	Block Na^+ flux through channel pore
2	Batrachotoxin Veratridine Aconitine	Stabilize open state
3	α-Scorpion toxins Type-1 sea -anemone toxins	Delay channel inactivation
4	ω-Scorpion toxins	Enhance channel activation
5	Brevetoxins Ciguatoxins	Cause persistent activation

ligand that binds to the site of interest. But such an approach is not generally possible in HTS programmes, as the binding site linked with the desired modulatory effect is often unknown, unspecified or a high-affinity ligand is not available. Approaches involving noncompetitive displacement of a radioligand through allosteric interactions with other sites may be substituted, although many potentially useful compounds will be missed if there is either no or little allosteric modification. These limitations mean that in essence only very few voltage-gated ion-channel targets are suitable for radioligand binding in HTS and usually other approaches are required.

Cell-based fluorescence and radiotracer assays

Ion-channel function may also be monitored through the measurement of changes in intracellular concentration of

permeant ions by using fluorescent-ion indicators or radiolabelled ions. Both approaches lend themselves to HTS in cell-based, 96-well formats. In these assays, ion channels are generally activated using toxins or compounds that promote prolonged channel opening, or if applicable to the channel type, high K^+ depolarization or elevation of intracellular Ca^{2+}. For example, Na^+ channels, which completely inactivate in <10 ms after voltage stimulation, can be induced into an open, ion-conducting conformation over many minutes using toxins such as veratridine or scorpion venom[8,9] (Figure 2). The Ca^{2+} channels exhibit no or a lesser degree of inactivation and hence can be opened by high K^+ depolarization as an alternative to using compound activators. Calcium-activated K^+ channels can be opened following addition of Ca^{2+} ionophores or as a consequence of Ca^{2+} influx through Ca^{2+} channels.

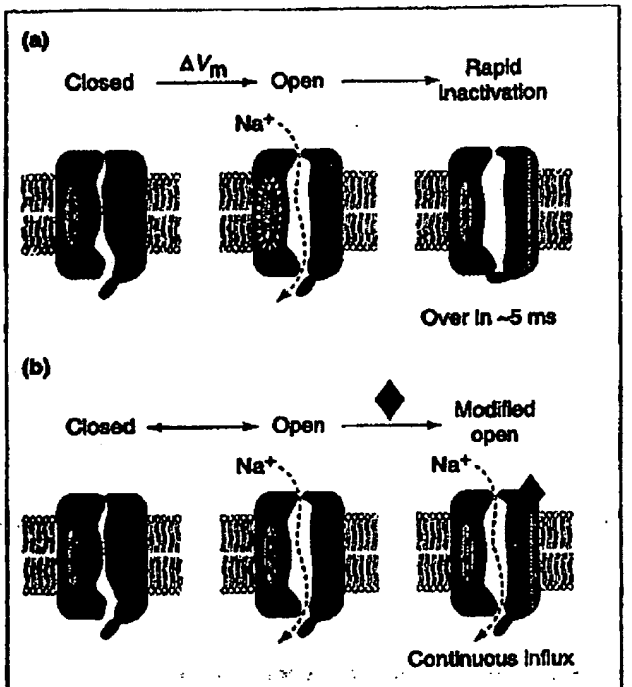

Figure 2. Na^+ channels can exist in multiple ion conducting (open) and nonconducting (closed/inactivated) conformations. (a) Na^+ channels open and then rapidly inactivate following voltage stimulation. Transitions between these states occurs in a voltage and time-dependent manner. The time course and voltage dependency of Na^+-channel activity can be described by separate activation and inactivation gating processes. Activation takes place upon depolarization of the membrane (ΔV_m) and the channel adopts an open pore conformation allowing Na^+ influx. Inactivation processes then change the channel conformation to a nonconducting, non-activatable state. Repolarization returns the channels from inactivated to resting conformations. (b) Na^+ channel opening may be prolonged by toxin binding. Toxins such as veratridine and batrachotoxin bind to channels in the open conformation and stabilize the channel in a modified conducting state. This in effect removes or slows down the inactivation process allowing ion flux to continue from minutes to hours.

Fluorescence readout is widely used for Ca^{2+} channels, as influx of Ca^{2+} through open channels causes large transient changes in intracellular Ca^{2+} levels (typically 100–1,000-fold) that can be detected using a range of commercially available fluorescent Ca^{2+} dyes such as Fluo-3 and Calcium green-1 (Molecular Probes, Eugene, OR, USA). In these assays, Ca^{2+} channels can be activated by depolarizing the membrane with an isotonic solution containing a high concentration of K^+. The resulting transient movement of intracellular Ca^{2+} can be measured over a duration of 20–60 s.

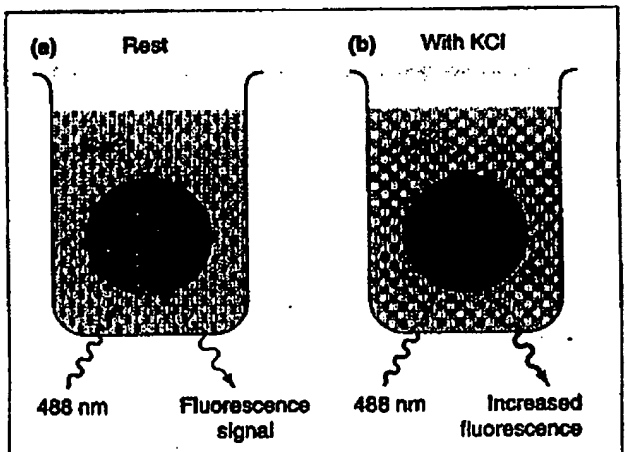

Figure 3. Schematic representation of a fluorescence-based 96-well Ca^{2+}-channel assay. Cells, loaded with the fluorescent Ca^{2+} indicator Fluo-3AM (a), are depolarized by addition of isotonic KCl (b). The depolarization activates the Ca^{2+} channels allowing Ca^{2+} entry and increases the fluorescence signal. Note that the single cell represents a cell monolayer or suspension.

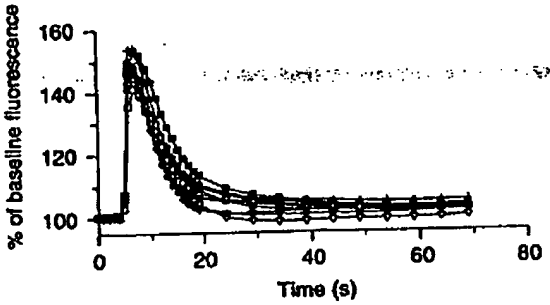

Figure 4. Fluorescence recording of Ca^{2+} responses in human neuroblastoma cells. Data from assay scheme described in Figure 3 run with adherent cell monolayers in a 96-well plate. Transient rises in fluorescence are recorded in the FLIPR system and show high reproducibility from well to well (time courses in signal from wells A1 to A12 are shown superimposed).

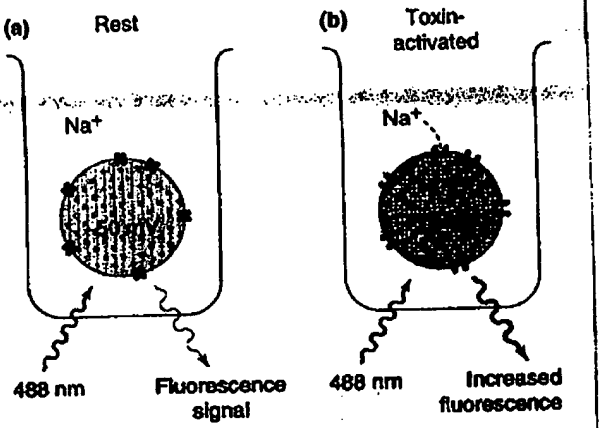

Figure 5. Use of bis-oxonol membrane potential dyes in Na^+-channel assays. Cells expressing Na^+ channels are bathed in media containing negatively charged bis-oxonol dye. At rest (a), the cells are hyperpolarized and the negative potential opposes dye entry. When Na^+ channels are continuously opened by the addition of an activating toxin (b), influx of positively charged Na^+ occurs and depolarizes the cell. A more positively charged cell leads to dye accumulation and hence an increase in fluorescence signal from the cell layer. Note that the single cell represents a cell monolayer.

The measurement of rapid kinetic changes in fluorescence for HTS purposes has recently become possible with the availability of a charge-coupled device (CCD)-based fluorescence plate reader equipped with integral 96-well pipettors, capable of reading 96 wells simultaneously at rates as fast as one plate per second. A typical Ca^{2+}-channel assay performed in-house measuring the transient movement of Ca^{2+} in a fluorescent-imaging plate-reader (FLIPR) system[10] (Molecular Devices, Sunnyvale, CA, USA) is shown in Figure 3 (assay scheme) and Figure 4 (kinetic data). Plate-throughput capacity for each assay is limited by the reading time and the time taken to change pipette tips; for a typical assay measuring the Ca^{2+} response over one minute, each plate is processed approximately every three minutes.

The FLIPR system[10] has optics designed to discriminate fluorescence from the cell monolayer and precise temperature control, enabling the slow membrane-potential-sensitive dye – bis-(1,3-dibutylbarbituric acid)trimethine oxonol [DiBAC(4)$_3$] – to be used in cellular ion-channel assays[11]. DiBAC(4)$_3$ is negatively charged and undergoes potential-dependent distribution between the cell cytoplasm and the extracellular medium. DiBAC(4)$_3$-based Na^+-channel FLIPR assays have also been developed in-house with cells expressing Na^+ channels, and using scorpion venom or veratridine to promote prolonged channel opening (Figure 5). Toxin activation of Na^+ channels causes resting cells to depolarize, which leads to a slow accumulation of dye in the cytoplasm and an increase in fluorescence signal. A similar approach can be used to follow K^+-channel activation through hyperpolarization and dye depletion[11,12]. The disadvantages of using the oxonol dyes are that they can only be used for slow membrane-potential changes that occur during the redistribution of dye between the cytoplasm and extracellular medium over a timescale of minutes rather than seconds. Furthermore, several test compounds directly influence the 'fluidity' of the cell membrane, leading to an increase in intracellular dye concentration and a false-positive response.

Radiotracers have long been used to follow ion flux through channels in cells and synaptosomal preparations. The radiotracers $^{22}Na^+$ and [^{14}C]-guanidinium are commonly used for pharmacological and toxicological analysis of Na^+ channels, and in compound development programmes[12,13]. Similarly, Ca^{2+}-channel activity has been measured in synaptosomes and cells following KCl depolarization using $^{45}Ca^{2+}$ as the radiotracer[14]. Assays for

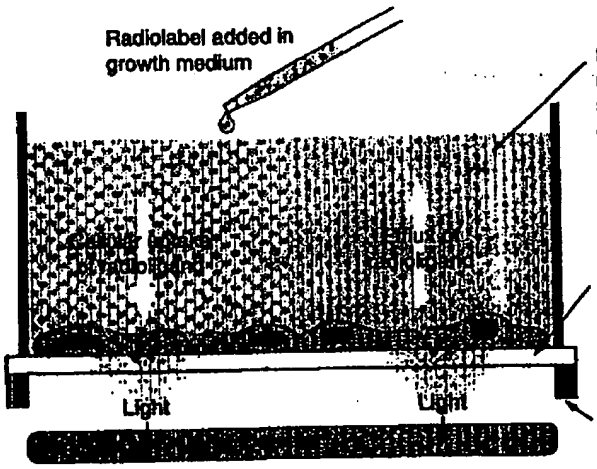

Figure 6. Principles of Cytostar-T scintillating microplate technology. Cytostar-T plates are standard format tissue culture-treated plates in which the transparent base of each well is a composite of polystyrene and scintillant that permits cultivation and observation of adherent cell monolayers. Radioisotopes that have suitable decay characteristics interact with the scintillant when brought into close proximity with the base of the plate by virtue of the biological process within the cells, thereby resulting in the generation of a light signal.

K^+-channel activity are possible using ^{86}Rb as the K^+-channel permeant ion. One application in glioma cells follows Ca^{2+}-activated K^+-channel activity through ^{86}Rb influx following addition of the Ca^{2+} ionophore ionomycin[15] – it is a useful technique for screening toxins and crude venoms for Ca^{2+}-activated K^+-channel activity.

Recently, the development of Cytostar-T scintillating microplates (Amersham International, Little Chalfont, UK) has enabled some radiotracer-flux assays to be performed much more efficiently and at higher throughput than protocols involving liquid scintillant, because the need for separation and washing is reduced[16] (Figure 6). A cell-based Na^+-channel assay has been designed in-house to take advantage of these plates. Chinese hampster ovary cells that are stably over-expressing a Na^+ channel are cultured within the Cytostar-T plates to form a confluent monolayer. Scorpion venom and veratridine are added to the cells to activate and maintain the Na^+ channels in an open state. Cells are loaded with ^{14}C-labelled guanidinium by addition to the well – this radiolabel is approximately the same physical size as Na^+ and passes through the open channel into the cell. After a fixed time period to allow the accumulation of radiolabel within the cytoplasm, the open Na^+ channel is blocked with tetrodotoxin. By virtue of the biological process, the accumulated radioactivity within the cell is brought into close proximity with the scintillant incorporated into the base of the plate, causing the generation of light that is measured using a scintillation counter. Test compounds that inhibit the opening of the channel, or block the pore, will inhibit the movement of radiolabel into the cell. With this technique, scintillation fluid is not required and no separation of the incorporated cellular radiolabel from that in the assay buffer is needed, because the confluent monolayer and the cell membrane prevent the non-incorporated radiolabel from generating a light signal. Assays developed in these plates are very suitable for automation and HTS (see below).

Cell viability

Cell viability assays using both yeast and mammalian cell lines have also been developed for ion-channel targets and may have potential for use in HTS to identify novel ligands[17–19]. In these assays, ion-channel activity and the flux of a particular ion is directly related to cell survival. Yeast-based assays employing K^+-uptake-defective *Saccharomyces cerevisiae* strains have been widely used for both expression cloning and structure–function analysis of plant inward-rectifier K^+ channels[19]. In these assays, expression of functional K^+ channels restores K^+ uptake and promotes cell survival. A screen based on this approach may prove to be valuable for identifying K^+-channel blockers in high-throughput screens.

A semi-automated assay for detecting and quantifying three classes of marine Na^+-channel toxins has also been developed using mammalian neuroblastoma cells with a colorimetric cell viability readout[18]. Here, cells are treated with a Na^+-channel opener (veratridine) and a Na^+/K^+-pump inhibitor (ouabain, which blocks Na^+ efflux), to potentiate a lethal intracellular Na^+ overload. Cell viability is enhanced by toxins that block the channel and is

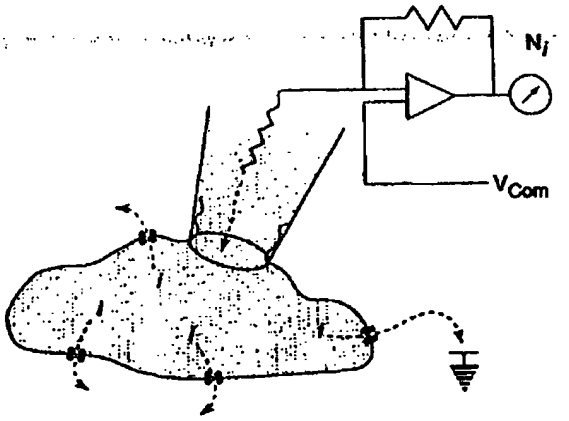

Figure 7. Patch clamp technique. The whole-cell mode of the patch clamp technique enables the sum of all the individual ion-channel currents to be recorded from the entire cell. Glass pipettes are sealed onto a cell membrane and the membrane patch ruptured to allow electrical access to the whole of the cell membrane. Patch clamping is the most sensitive technique for recording ion-channel activity and allows full voltage control of the cell membrane. Single cells are required for this technique. N denotes the number of channels, i the single channel current, hence N is the total current measured through N channels. V_{Com} denotes the command voltage applied via the pipette to the interior of the cell.

reduced by toxins that further promote Na^+-channel opening. This screen was proposed as a useful adjunct to reduce the need for *in vivo* toxicity testing of seafood products in mice. The assay protocol has recently been developed further for contract routine testing of paralytic shellfish poisons (MIST bioassay, Jellett Biotek, Dartmouth, NS, Canada)[20].

Electrophysiology

Electrophysiological voltage-clamping techniques encompass the most powerful approach for detailed biophysical analysis of ion-channel function through measurement of ionic current flowing through one or many ion channels. Patch clamping uses a single microelectrode for controlling the membrane voltage whilst measuring the current flow through a single cell or membrane patch[21] (Figure 7). Since the development of this technique, our understanding of ion-channel function and mechanisms of drug action have moved forward rapidly. However, along with other voltage clamp methodologies, patch clamping has not yet evolved into a high-throughput process for compound screening. Perhaps the best solution to date for using patch clamping in a screening programme has been designed around an automatic compound delivery system that uses HPLC autoinjectors coupled with on-line data sampling. NeuroSearch A/S (Glostrup, Denmark) have used this approach in their large-conductance Ca^{2+}-dependent K^+ (BK)-channel screening programme, enabling many compounds to be studied in excised patches from transfected HEK cells[22]. Further automation of the patch clamping process would further improve throughput capacities.

HTS program

How would these kinds of technique fit within a typical screening programme? For a high-throughput campaign (200,000 sample), binding assays remain the first choice in terms of cost, automation and throughput rate. This reflects the technical ease of these types of assay and, hence, their ability to be automated. The types of screening sample will vary and will usually include corporat compound collections, natural product extracts and combinatorial libraries presented either discretely or as pools.

Cellular functional assays are used as primary or secondary assays to determine functionality of compounds from a binding screen and also to assess toxicity. As cellular functional screens are typically more labour intensive, at Glaxo Wellcome we screen around 50,000 samples in an equivalent time-frame to a 200,000 sample binding assay. However, these types of assays are information rich and therefore potentially offer greater rewards to the drug hunter. On-going developments in cellular-screening automation and compatibility with 384-, 864- and 1,536-well plates (in terms of cell plating, reagent addition and detection) are expected to increase the throughput of cellular assays to match that of binding assays. At the higher densities (864- and 1,536-well plates), plate washing of adherent cells is more problematic.

Patch clamping remains an important tertiary assay in a hits-to-leads programme, yielding information about voltage, rate- and use-dependence of compound binding. Throughputs here are very low, at best in the order of 10–20 compounds per day, depending on the assay protocol.

Chemistry

Over the past few years a massive amount of effort has been put into the design of combinatorial chemistry

libraries and technology for use in HTS. Although most libraries finding their way into ion-channel screens will undoubtedly be those used in any other target screens (that is, a diverse set of small molecules designed to probe as much molecular space as possible), there has been some interest in designing libraries around small-molecule structural units commonly associated with ion-channel modulation – such as dihydropyridine libraries for Ca^{2+}-channel blockade[23,24], dihydrobenzopyran libraries for K^+-channel openers[25] and biphenyl-derivative libraries for K^+-channel blockade[26]. Other workers have prepared peptide libraries drawing lessons from the multitude of bioactive venom peptides[27,28].

Automation

Technological advancements are being made in automation and miniaturization of HTS in order to allow larger sample capacities per day at reduced cost. Currently, there are two approaches to HTS automation: the integrated systems and the work stations. The relative merits of these approaches have been described elsewhere[29]. In Glaxo Wellcome, the HTS operation is based around integrated robotic systems that handle assays in 96-well and 384-well plates[29]. The challenges for ion-channel screening using this type of automation are the design of robot-compatible assays and integration of readers like FLIPR into these robotic systems. Once achieved, full automation will enable 24 h continuous operation without requiring shift-work and allowing assays to be performed more efficiently and economically. For example, the Na^+-channel assay utilizing Cytostar-T plates, described above, has been automated for screening in-house using the Beckman (Fullerton, CA, USA) Biomek 2000 plus side-loader fully integrated to a Wallac (Turku, Finland) Trilux Microbeta scintillation counter. This approach has significantly increased the throughput of the assay, while reducing the degree of practical work involved.

Future technologies

A key issue in developing new high-throughput approaches for voltage-gated ion channels is designing equipment and techniques by which rapid and repetitive changes in membrane potential can be induced in a 96-well or higher density formats.

For relatively slow responses, such as intracellular ion accumulation, electrical-field stimulation through extracellular electrodes may be useful for assaying rate- and use-dependent effects of drugs on ion channels. Current technologies that may be applicable to this area include planar microelectrode arrays designed for stimulating and recording from neuronal networks in long-term culture[31]. Such arrays lend themselves to high-density format designs.

For fast membrane-potential responses, recording through extracellular electrodes is possible[32] but yields signals that are very difficult to interpret in terms of ion-channel activity alone. Thus, alternative recording techniques are required that will match intracellular electrical electrodes in terms of potential waveform discrimination and allow analysis of ion-channel activity. It is possible that the development of optical recording systems for use with fast membrane-potential dyes will provide a way forward.

Recent emerging ion-channel technologies have been described in the field of biosensors. Ion channels (gramicidin) incorporated into artificial membranes and linked to antibodies are being used to detect receptor–ligand interactions[33]; here, ionic currents only pass when partners of a dimeric channel align with each other. These ligand–receptor interactions tether the gramicidin molecules, preventing pore formation and thus reducing current flow. Such techniques lend themselves immediately to highly miniaturized formats, given the high resolution and sensitivity of current measurement circuitry. But whether the development of this type of technology is directed towards ion-channel assays *per se* remains to be seen.

Conclusions

Various technologies and screening approaches are evolving to meet the requirements for voltage-gated ion-channel HTS. Current techniques that can be translated into 96- and 384-well plate format are all associated with a lack of precise voltage control, so that information regarding voltage, use- or rate-dependence remains unknown. This is frustrating given the advantages offered by state- or rate-dependent ion-channel modulators. Patch clamping, although allowing such precise dynamic voltage control of channel state is by no means a technique for HTS. The incorporation of dynamic voltage control into microtitre plate assays therefore remains a key challenge in the ion-channel technology arena.

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In short...

DuPont announced on 19 May an agreement to acquire **Merck & Co.**'s interest in their joint venture – The DuPont Merck Pharmaceutical Co. – which was formed in 1991. The $2.6 billion transaction is expected to be completed in July when DuPont Merck will become an integral part of DuPont and operate under the name **DuPont Pharmaceuticals**. Charles O'Holliday, Jr, President and CEO of DuPont, said that 'this action will enable us to more fully integrate our materials and life science research platforms'. The acquisition is the third major action that DuPont has made recently to strengthen its life sciences portfolio. Kurt M. Landgraf, DuPont Executive Vice President, says that 'the acquisition of Protein Technologies International and the formation of Optimum Quality Grains joint venture with Pioneer Hi-Bred International have made DuPont a leader in agricultural biotechnology. And now we have added the biotechnology potential of a wholly owned pharmaceutical company'.

Oxford Molecular aspires to be the world leader in supplying drug discovery research services. As part of this goal, the acquisition of **Chemical Design** and its Chem-X software will provide a broader range of product offerings to scientists in chemoinformatics, molecular design and combinatorial chemistry. For Oxford Molecular's customers it will mean that the right compounds can be brought through the drug discovery process faster and at lower cost. The company will also have the opportunity to integrate the acquired products with selected Oxford Molecular products to provide more comprehensive software solutions for discovery research scientists.

Therapeutic Antibodies Inc. and **G.D. Searle & Co.**, the pharmaceutical division of **Monsanto Co.**, announced on 21 May the signing of a research collaboration agreement for the identification, development and marketing of a new antibody-based drug. Therapeutic Antibodies will develop, manufacture and register a highly purified polyclonal antibody for a target indication nominated by Searle. It is forecast that Searle will pay US$8 million in R&D payments and product supplies based on achieving certain milestones. The first milestone payment of $1 million was made on signing. While Searle anticipates having the worldwide marketing rights of the product, Therapeutic Antibodies will be responsible for the ongoing supply. There will be shared revenues from the successful commercialization of the product.

Exelixis Pharmaceuticals and **Bayer AG** have entered into a collaboration to identify novel screening targets for the development of new crop protection agents. The collaboration will bring together Exelixis' expertise in model system genetics, genomics and bioinformatics and Bayer's experience in the development and commercialization of products for the agricultural market. Exelixis may receive up to $30 million in license fees, research support and milestone payments based on program success, and will receive royalties paid on Bayer's sale of any product arising from the collaboration.

Exelixis will also develop a novel EST database for a pest species of strategic importance to Bayer; assays developed by Exelixis will be used by Bayer to screen against its extensive libraries of chemical compounds, evaluate lead structures *in vivo* and develop and commercialize crop protection products. 'We believe that biology-based approaches to pesticide discovery being pioneered by Exelixis will identify novel targets that will lead to new highly effective crop protection agents', says Georg Scangos, President and CEO of Exelixis.

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FEE TRANSMITTAL for FY 2003

Effective 01/01/2003. Patent fees are subject to annual revision.

☒ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 55

Complete if Known	
Application Number	09/456,429
Filing Date	December 8, 1999
First Named Inventor	Yu-Hua Ji
Examiner Name	M. Garcia Baker
Art Unit	1639
Attorney Docket No.	P-015-RP1

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☒ Deposit Account:

Deposit Account Number: 50-0344

Deposit Account Name: Theravance, Inc.

The Director is authorized to: *(check all that apply)*
☒ Charge fee(s) indicated below ☒ Credit any overpayments
☒ Charge any additional fee(s) during the pendency of this application
☐ Charge fee(s) indicated below, **except for the filing fee** to the above-identified deposit account.

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	750	2001	375	Utility filing fee	
1002	330	2002	165	Design filing fee	
1003	520	2003	260	Plant filing fee	
1004	750	2004	375	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	

SUBTOTAL (1) ($) 0

2. EXTRA CLAIM FEES

			Extra Claims		Fee from below		Fee Paid
Total Claims		-20 **	0	X		=	0
Independent Claims		-3 **	0	X		=	0
Multiple Dependent				X		=	0

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	84	2201	42	Independent claims in excess of 3
1203	280	2203	140	Multiple dependent claim, if not paid
1204	84	2204	42	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0

***or number previously paid, if greater; For Reissues, see above*

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet.	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	55.00
1252	410	2252	205	Extension for reply within second month	
1253	930	2253	465	Extension for reply within third month	
1254	1,450	2254	725	Extension for reply within fourth month	
1255	1,970	2255	985	Extension for reply within fifth month	
1401	320	2401	160	Notice of Appeal	
1402	320	2402	160	Filing a brief in support of an appeal	
1403	280	2403	140	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive – unavoidable	
1453	1,300	2453	650	Petition to revive – unintentional	
1501	1,300	2501	650	Utility issue fee (or reissue)	
1502	470	2502	235	Design issue fee	
1503	630	2503	315	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17 (q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	750	2809	375	Filing a submission after final rejection (37 CFR § 1.129(a))	
1810	750	2810	375	For each additional invention to be examined (37 CFR § 1.129(b))	
1801	750	2801	375	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($) 55

SUBMITTED BY

Complete (if applicable)

Name (Print/Type)	Joyce G. Cohen	*Registration No. Attorney/Agent)*	44,622	*Telephone*	(650) 808-6000
Signature	Joyce Cohen			*Date*	May 13 2003

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This collection of information is required by 37 CFR 1.17 and 1.27. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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COPY

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